Toxicology and Pharmacokinetics of Intravesical Gemcitabine: A Preclinical Study in Dogs

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INTRODUCTION

It is estimated that the incidence of bladder cancer in the United States in 1999 will be ~54,200 new cases with an expected mortality of 12,100 (1). In men, bladder cancer remains the fourth most common malignancy and will account for 6% of all cancer cases, whereas in women, it is the eighth most common malignancy and will account for 3% of all cancers (1). Furthermore, the incidence of bladder cancer has increased in the United States by 36% from 1956 to 1990 (2). Presently, 74% of bladder cancers are superficial at initial diagnosis, 18% are advanced but confined to the pelvis, whereas only 3% have metastasized (2). However, up to 30% of all patients with superficial disease will develop muscle invasive tumors within 5 years (3, 4).

Treatment of superficial disease aims to eradicate existing disease and to prevent tumor recurrence and/or progression to muscle invasion and metastasis. Patients treated by transurethral resection alone have only a 49% chance of remaining free of disease recurrence (5). Intravesical chemotherapy and immunotherapy have resulted in a significant reduction in the risk of disease recurrence. Superficial bladder cancer lends itself to intravesical therapy due to the potential for direct contact between chemotherapeutic drug and tumor. Furthermore, very high concentrations of agents can be achieved in the bladder with minimal systemic toxicity.

Intravesical immunotherapy with BCG is presently the most effective agent for the therapy and prophylaxis of superficial bladder cancer (5–9). A randomized trial comparing BCG to no intravesical treatment has shown 10-year progression-free rates of 62% for BCG-treated patients compared with 37% for control patients (10). However, with longer follow-up in this series, it became clear that patients with high-risk superficial bladder cancer treated with BCG are at life-long risk of progression and metastasis (11). Unfortunately, intravesical BCG is associated with serious morbidity and even mortality (6, 12).

More active chemotherapy agents are needed for patients who fail BCG. Present intravesical chemotherapeutic agents for the treatment of bladder cancer that have been well studied in human trials include thiopeta, doxorubicin, Mitomycin C, and epirubicin (5, 13, 14). Overall, intravesical chemotherapy in the treatment of carcinoma in situ has been shown to achieve complete response rates ranging from 34 to 53% (5, 13, 14). Furthermore, intravesical chemotherapy has been shown to achieve eradication of residual disease and prophylaxis of recurrent disease in 48 and 14% of patients, respectively (13). However, no statistically significant reduction in the risk of disease progression has been shown in >2000 patients enrolled.
in prospective, randomized, controlled trials of thiopeta, doxorubicin, Mitomycin C, and epirubicin (13). These chemotherapeutic agents are generally well tolerated and devoid of serious side effects (14). Mitomycin C, which has a 12–40% partial response rate when given systemically for bladder cancer, is one of the most active intravesical agents (5). However, in 1157 patients treated in six studies, the overall reduction in recurrence rate after mitomycin was only 15% (5). Presently, no single agent is available for intravesical therapy that has demonstrated a complete response rate when administered systemically for metastatic bladder cancer. Therefore, there is a compelling need for more effective and well-tolerated chemotherapeutic agents for the intravesical treatment of bladder cancer.

Gemcitabine is a novel deoxycytidine analogue with a broad spectrum of antitumor activity that was recently approved in the United States for treatment of pancreatic cancer (15, 16). Gemcitabine has a molecular weight of 299.66, and after intracellular activation, the active metabolite is incorporated into DNA, resulting in inhibition of further DNA synthesis. Gemcitabine may also inhibit ribonucleotide reductase, stimulate deoxycytidine kinase, and inhibit cytidine deaminase as part of its cytotoxic activity (15).

Gemcitabine has been shown recently to be highly effective and well tolerated when given systemically for the treatment of metastatic transitional cell carcinoma (17–20). Two Phase II trials in previously untreated patients have shown overall response rates of 24.3 and 28% in 37 and 39 patients, respectively, who received 1200 mg/m² weekly for 3 weeks in a 4-week cycle (17, 18). A recent Phase II trial of patients, respectively, who received 1200 mg/m² weekly for 3 weeks in a 4-week cycle (17, 18) showed overall response rates of 24.3 and 28% in 37 and 39 patients treated with 1200 mg/m² of gemcitabine found an overall response rate of 35 previously treated patients with metastatic bladder cancer (17, 18). A recent Phase II trial of patients, respectively, who received 1200 mg/m² weekly for 3 weeks in a 4-week cycle (17, 18) showed overall response rates of 24.3 and 28% in 37 and 39 patients treated with 1200 mg/m² of gemcitabine found an overall response rate of 22.5% (four complete and three partial responses; Ref. 19). These results in patients who had failed one prior cisplatin-containing regimen suggest that gemcitabine may be very useful for patients who have failed standard chemotherapeutic regimens (19). The drug was well tolerated in all of these studies, with a low incidence of systemic side effects. Gemcitabine, therefore, represents a logical candidate for intravesical therapy.

No preclinical safety data exist for intravesical administration of gemcitabine, a necessary prerequisite for clinical trials. This study was designed to determine whether intravesical instillation of gemcitabine was safe, to assess for organ-specific toxicity and to identify whether systemic absorption of the drug occurs when instilled into the bladder.

**MATERIALS AND METHODS**

Full Animal Care and Use Committee approval was granted before the study was undertaken. Twelve beagle dogs were used for this study because the dog bladder closely resembles the human bladder on histological examination (21). The dogs were obtained from Summit Ridge Farms (Susquehanna, PA) and confirmed to be in good health by veterinary scientists at Memorial Sloan-Kettering Cancer Center. They were housed in stainless steel cages and fed Purina Dog Chow and water ad libitum. Premedication for treatment was accomplished with atropine (Henry Schein, Port Washington, NY) 0.02 mg/lb s.c., followed by sedation with a mixture of 10 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 0.4 mg/kg i.v. diazepam (Steris Laboratories, Inc., Phoenix Arizona).

For intravesical therapy, each animal was catheterized with an 8F Rob-Nel catheter (Sherwood Medical, St. Louis, MO), and the bladder was emptied. The animals then received gemcitabine in various doses dissolved in 50 ml of normal saline, and the catheter was clamped for 1 h. Blood samples were drawn by venipuncture of the cephalic vein at designated time intervals, to which 1 mg of THU (Calbiochem-Novabiochem Corp., La Jolla, CA) was immediately added to prevent ex vivo degradation of gemcitabine by cytidine deaminase in the serum. Samples were placed on ice until centrifugation at 3000 rpm for 20 min. Isolated serum was stored at −20°C until analysis. The animals recovered after therapy, and venipuncture was completed and inspected daily for signs of toxicity. Mean weight of the animals was 8.0 kg, with an estimated surface area of 0.40 m². Using a Kₐ factor of 20, it was determined that 350 mg of drug for these animals would be equivalent to the human dose of 1000 mg/m² (20). The 1000 mg/m² dose is the usual systemic dose for human Phase II trials.

**Toxicology**

Animals (n = 6), in groups of two, received 100 mg, 350 mg, or 1 g of drug on alternate days three times per week for 4 weeks. Blood was taken for peripheral blood counts and gemcitabine assay with each instillation immediately prior to the bladder being drained at the completion of each treatment. Animals were inspected daily for signs of distress. At the end of the 4-week treatment schedule, one dog from each group was euthanized at day 1 and one at day 14 after treatment. A full necropsy was performed to assess acute and subacute toxicity in each group. Particular attention was paid to a bone marrow aspirate and trephine and any treatment-related effect on the bladder.

**Pharmacokinetics**

**Intravesical Administration.** Animals (n = 6) were initially treated with 350 mg (n = 1) and 3.5 g (n = 1) of drug, and blood was taken at 30, 60, 120, 240, and 480 min after instillation. Very high serum levels of drug were seen in the animal treated with 3.5 g of drug, and the experimental protocol was modified because of the clinical toxicity observed in this animal. Subsequent animals were treated with 350 mg (n = 2) and 100 mg (n = 2), and blood samples were drawn at the above time points with the addition of extra samples taken at 75, 90, and 105 min after instillation.

**i.v. Administration.** Two dogs were treated with 350 mg of drug by i.v. infusion over 1 h. Blood samples were drawn at the above designated time intervals, and all samples were treated and stored as described above.

**Assessment of Gemcitabine Degradation in Urine.** Dog (n = 2) and human urine (n = 1) was examined to evaluate for degradation of gemcitabine. Prior to gemcitabine treatment, catheterized urine was obtained and stored to be examined later as a negative control. After the first treatment of both animals in the 100-mg group, urine was taken and treated with or without...
THU. Two samples of human urine were collected from a healthy volunteer with no evidence of proteinuria, and gemcitabine was added to make an approximate concentration of 1 mg/ml. These samples were incubated overnight with and without THU to examine for any ex vivo degradation of the drug in urine.

Assessment of Gemcitabine Levels in Dogs Undergoing Toxicology Experiments. Blood was taken for gemcitabine assay, with each instillation of gemcitabine in the animals undergoing toxicology studies. The samples were collected 1 h after each instillation had begun. These assays were undertaken to determine whether detectable levels of drug were present in the serum after repeated treatments.

High-Performance Liquid Chromatography Methodology

The standard was prepared by adding control plasma containing THU before sample preparation. For sample preparation, 20 µl of 2-propanol were added to 1 ml of plasma. The plasma was placed in a Millipore Ultrafree-MC filter unit, and refrigerated centrifugation was performed. Twenty µl of the ultrafiltrate were used for analysis without further modification. The high-performance liquid chromatography analysis was performed with an Inertsil 5 µm ODS-3 4.6 × 250 mm column with a mobile phase of 2% acetonitrile/50 mM ammonium carbonate with 0.1% triethylamine at pH 9. The effluent was monitored at 265 nm, with a retention time of gemcitabine of about 18 min and instrument cycle time of

Fig. 1 Bone marrow from an animal after treatment with 350 mg (three times weekly for 4 weeks) of intravesical gemcitabine showing normal cellularity with a normal myeloid:erythroid ratio.
50 min. The standard curve was linear from 0.15 to 10 µg/ml with sensitivity to 0.1 µg/ml ($S/n = 3$).

Statistical Methods

The AUCs for each series of data were assessed using the Simpson’s and Trapezoidal rules (Pharmacological Calculation System-version 4.2 (A)). Half-lives ($T_{1/2}$) for each series of data were calculated using RSTRIP II software (version 2.0 © 1993; Micromath, Inc.). The Paired Samples t test was used to determine differences in $T_{1/2}$ values between groups, with $P < 0.05$ considered statistically significant.

RESULTS

Toxicology

The 100- and 350-mg doses of intravesical gemcitabine were well tolerated with no clinically demonstrable side effects. No fall in platelet or white cell count was observed in the 100-mg group. A slight drop in platelet count and white cell count was seen in the 350-mg group, with a white cell nadir of 2.9 and 3.1 (x10$^3$/µL) and a platelet nadir of 97 and 63 (x10$^3$/µL). The mean ($±$SD) white cell count and platelet count for normal, sexually mature, male beagle dogs ($n = 243$) is 11.5 ($±$3.3 $×$ 10$^3$/µL) and 405 ($±$ 113 $×$ 10$^3$/µL, respectively (data on Fig. 2 Bone marrow from an animal after treatment with 1 g (three doses) of intravesical gemcitabine showing severe hypocellularity.
file; Summit Ridge Farms). The nadirs seen in this study were observed toward the end of the 4-week treatment period in both animals, with rapid recovery after the cessation of treatment in the animal euthanized at day 14. At necropsy in both groups, no toxicity was noted in these animals. In particular, no toxicity was observed in the bladder and the bone marrow. Examination of the bone marrow in both groups revealed normal cellularity with a normal myeloid:erythroid ratio (Fig. 1).

The animals receiving 1 g of drug demonstrated severe toxicity and received only three doses before treatment was discontinued. A white cell nadir of 1.8 and 0.2 (x10^3/μl) and a platelet nadir of 141 and 78 (x10^3/μl) was observed in these animals. One animal died from small intestinal hemorrhage 2 days after the last treatment. The other animal was euthanized 5 days after treatment because of febrile neutropenia unresponsive to treatment with i.v. antibiotics and i.v. fluids. Necropsy in these animals revealed multiorgan toxicity including severe bone marrow hypoplasia (Fig. 2), transmural hemorrhage and necrosis in the small intestine (Fig. 3), and severe ulcerative and hemorrhagic cystitis (Fig. 4).

**Pharmacokinetics**

**Intravesical Administration.** Peak serum levels of gemcitabine were detectable at 30–60 min, with detectable levels at 8 h after instillation in all animals. The initial experiment (Fig. 5) revealed a peak level of 517.25 μM at 30 min and a prolonged T1/2 of 273.6 min in the animal that received 3.5 g of drug. The animal treated with 350 mg of drug had a peak level of 29.90 μM.
at 60 min with a $T_{1/2}$ of 334.4 min after treatment. After these results, the experiment was modified to address the pharmacokinetics of lower doses of drug, and blood samples were taken at more frequent time intervals.

Additional animals were evaluated after receiving 350 mg ($n=2$) and 100 mg ($n=2$), with blood samples drawn at more frequent time points (Fig. 6). Three animals in total were therefore evaluated at the 350-mg dose (Fig. 7). Detectable serum levels up to 8 h after intravesical administration of gemcitabine were demonstrated in these animals. The blood samples were taken at more frequent time points than the initial experiment. The $T_{1/2}$s were 321.0, 334.4, and 329.8 min for the 350-mg animals compared with 274.9 and 236.2 min for the 100-mg animals. One animal in the 350-mg group had a low peak serum level (8.31 $\mu$M) compared with his experimental cohort (34.11 $\mu$M) and compared with the data from the equivalent dose animal in the initial experiment (29.90 $\mu$M). The low peak serum level may have been due to leakage of gemcitabine around the urinary catheter, which was noted to occur in this animal at the time of instillation. Data from this animal was not used for comparison of AUCs.

**i.v. Administration.** Two animals (one each from the 100- and 350-mg groups above) received an i.v. infusion of 350 mg of gemcitabine over 1 h. These animals demonstrated very similar peak serum levels (79.76 and 79.49 $\mu$M at 60 min) and a very similar AUC (3659 and 3732 $\mu$g/ml/min). The $T_{1/2}$ result was 103.0 and 95.6 min for these animals. The mean ($\pm$SD) $T_{1/2}$ for animals receiving 350 mg i.v. was 99.3 ($\pm$5.2) min com-

Fig. 4 Histological section of bladder from an animal after treatment with 1 g (three doses) showing hemorrhage and cystitis.
pared with 328.4 (±6.8) min for animals receiving 350 mg of intravesical gemcitabine. This difference was highly statistically significant (P < 0.001). The mean (±SD) for the AUC after i.v. administration was 3695.5 (±51.6) compared with 1819 (±124.5) for intravesical administration. This result is also highly statistically significant (P < 0.001). It is clear from this data that both the T₁/₂ and the AUC are significantly elevated after intravesical instillation of the equivalent systemic dose of gemcitabine (Fig. 7).

Assessment of Gemcitabine Degradation in Urine. No significant degradation of gemcitabine occurred in the urine without THU in one animal and the human subject (Fig. 8). One animal had a small reduction in the absence of THU (8.63 to 5.89 mM). Both animals had very high levels of gemcitabine detectable in the urine drained from the bladder after treatment, indicating that the gemcitabine was not completely absorbed and that a biohazard exists in handling this fluid. Furthermore, the detectable level in the human urine approximated the known concentration of drug added (1 mg/ml), indicating that no ex vivo degradation of drug occurred overnight at room temperature in human urine.

Assessment of Gemcitabine Levels in Dogs Undergoing Toxicology Experiments. The median (range) levels of gemcitabine detected in the serum at 1 h after treatment in the 100-mg group were 3.10 μM (0.33–6.47) and 2.44 μM (0.27–11.65). The median (range) levels of gemcitabine detectable in the 350-mg group were slightly higher at 3.94 μM (1.18–9.58) and 3.27 μM (1.37–7.11). Although the variability of the detectable levels was substantial, every animal had detectable levels of gemcitabine in the serum, indicating that systemic absorption had occurred with every intravesical instillation.

DISCUSSION
Table 1 summarizes the data from this experiment, which we believe is necessary for human clinical trials of intravesical gemcitabine to be undertaken. We have demonstrated that gemcitabine instillation into the bladder does not result in urothelial toxicity. No bladder-specific toxicity was noted at doses that resulted in mild myelosuppression, as evidenced by a slight fall in platelet and white cell counts without evidence of bone marrow abnormality. Although cystitis was observed at the highest study dose, this toxicity occurred in the presence of multiorgan toxicity because of systemic absorption of excessive drug. These findings suggest that, at the appropriate dose, no organ-specific toxicity of the bladder would be expected.

The 350-mg dose, which is equivalent to the recommended human systemic dose of 1000 mg/m², was well tolerated with no adverse clinical effects and no demonstrable effect on the bone marrow or bladder. There was, however, evidence of mild myelosuppression, with a slight fall in platelet count and white cell count. These findings suggest that this dose is probably at the threshold above which increasing morbidity may occur. This observation also implies that the spectrum of toxicity at potentially therapeutic doses may be restricted to mild and uncomplicated myelosuppression.

This study demonstrated that significant systemic absorption of gemcitabine occurs after intravesical administration and that serum levels are detectable at up to 8 h after treatment. This
was true even for the lowest dose of 100 mg three times weekly for 4 weeks. We have demonstrated a markedly prolonged half-life after intravesical administration when compared with i.v. administration. The $T_{1/2}$ after i.v. administration demonstrated in this study (99.3 ± 5.2 min) compares favorably with previous investigators who have shown that gemcitabine is rapidly deaminated in dogs after i.v. administration and that the half-life is only 1.38 h (22). This rapid deamination has been shown to be due to significant pyrimidine nucleoside deaminase activity in the liver of the beagle dog (23). The enzymatic activity was similar to that seen in postnatal human liver, which makes the beagle dog an excellent animal model for this type of experiment (24). The $T_{1/2}$ after intravesical administration of the equivalent systemic dose was demonstrated in this study to be 328.4 ± 6.8 min, which is more than three times the value for i.v. administration. Furthermore, we have shown (Fig. 7) that for a given dose (350 mg), the AUC for intravesical administration is approximately one-half that observed with an i.v. infusion. Because the AUC is a better predictor of toxicity, it is concluded that the equivalent systemic dose if given intravesically would be likely to be one-half as toxic.

The prolonged $T_{1/2}$ and relatively large AUC are clearly important when designing human trials of this generally well-tolerated systemic agent. It is known that in humans, i.v. gemcitabine pharmacokinetics are linear and described by a two-compartment model and that volume of distribution and half-life are significantly influenced by duration of infusion (24). Clinically significant toxicity, defined as myelosuppression, has been seen in humans with weekly doses of 300 mg/m² at or above a 270-min infusion time (25). It is possible that, although the bladder was fully drained after each instillation, continued absorption of the drug may have occurred, with a resultant relative increase in toxicity. Furthermore, the low molecular weight of 299.66 of the compound may explain the absorption seen in this study as a similar phenomenon to that which has been observed with other low molecular weight intravesical chemotherapeutic agents such as thiotepa (26, 27).

The systemic absorption of drug and the prolonged $T_{1/2}$ are novel findings of this study and will impact on the design of clinical trials. Prolonged systemic levels may result in systemic side effects, particularly myelosuppression. However, due to the marked systemic activity of the drug in metastatic urothelial cancer, prolonged serum levels may represent a therapeutic advantage. Potentially greater drug levels within the bladder or regional tissues such as lymph nodes may correspond to a benefit in treating micrometastatic disease in these sites. It has been demonstrated that longer infusion times result in higher intracellular levels of gemcitabine triphosphate, an active metabolite responsible for cytotoxicity. The relatively high systemic levels also imply that substantial absorption occurs in the bladder, possibly more than the five- to seven-cell layer penetration typically seen with intracavitary chemotherapy. Deep penetration of the bladder wall with a systemically active chemotherapeutic agent raises the possibility that intravesical gem-

Table 1  Summary of both the toxicology and pharmacokinetics data for all animals

<table>
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<th>Dose</th>
<th>No. of animals*</th>
<th>AUC (±SD) μg/ml/min</th>
<th>$T_{1/2}$ (±SD) min</th>
<th>Peak level (±SD) μM</th>
<th>Toxicity</th>
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<td>100 mg</td>
<td>4</td>
<td>215.5 (±169.0)</td>
<td>255.5 (±27.4)</td>
<td>5.27 (±3.11)</td>
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<tr>
<td>350 mg intravesical</td>
<td>5</td>
<td>1819 (±124.5)</td>
<td>328.4 (±6.8)</td>
<td>18.59 (±21.95)</td>
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<tr>
<td>350 mg intravenous</td>
<td>2</td>
<td>3695.5 (±131.6)</td>
<td>99.3 (±5.2)</td>
<td>52.85 (±38.06)</td>
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<td>1 g</td>
<td>2</td>
<td>25764</td>
<td>273.6</td>
<td>106.77 (±42.50)</td>
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<td></td>
<td></td>
<td>517.25</td>
<td>Yes</td>
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</table>

* Two animals were reutilized for i.v. pharmacokinetic studies.

Fig. 8  Urine gemcitabine levels in two dogs taken pretreatment (pre Rx) and after treatment with (+THU) and without (−THU) THU and in one human subject incubated with [Human (+THU)] and without [Human (No THU)] THU overnight at room temperature.

[Table 1]

**A Preclinical Study of Intravesical Gemcitabine**
citabine could reduce the progression of superficial bladder cancer to muscle-invasive disease, a goal that has not been achieved with presently available chemotherapy agents.

This study has demonstrated that although significant systemic absorption of gemcitabine occurs after intravesical administration, it is possible to deliver a clinically active dose that does not result in bladder or systemic toxicity. On the basis of these data, a Phase I clinical trial will be conducted at doses up to 1000 mg/m².

REFERENCES

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